ARTHRITIS & RHEUMATISM
Vol. 41, No. 2, February 1998, pp 263–273

PRESENCE OF CARTILAGE-DERIVED MORPHOGENETIC PROTEINS IN ARTICULAR CARTILAGE AND ENHANCEMENT OF MATRIX REPLACEMENT IN VITRO

LUDWIG ERLACHER, CHEE-KENG NG, ROBERT ULLRICH, SIGURD KRIEGER, and FRANK P. LUYTEN

Objective. **To investigate the effects of the cartilage-derived morphogenetic proteins (CDMPs) in an in vitro cartilage explant model that mimics the chondrocytic response to matrix depletion, and to demonstrate their presence in articular cartilage.**

Methods. **Adult bovine articular cartilage and postmortem specimens from adult human donors with and without osteoarthritic (OA) lesions were stained by immunohistochemistry using polyclonal antibodies specific for CDMP-1 and CDMP-2. Extracts of bovine articular cartilage were analyzed by Western blotting for the presence of the CDMPs. Bovine articular cartilage explants were depleted of their matrix by trypsin digestion, followed by a 7-day culture period in a chemically defined serum-free basal medium (BM), with or without recombinant CDMPs 1 and 2. The metabolic activity of chondrocytes was measured by ^{35}S-sulfate incorporation into macromolecules. Newly synthesized proteoglycans (PGs) were analyzed using Sephacryl S-500 HR gel chromatography. The expression levels of the messenger RNA (mRNA) for chondrogenic markers were investigated by Northern analysis.**

Results. **CDMP-1 and CDMP-2 were detected in both bovine and human healthy and OA articular cartilage. Treatment of matrix-depleted cartilage explants with CDMPs 1 and 2 increased equally the incorporation of ^{35}S-sulfate into PGs compared with tissue maintained in BM. Gel chromatography analysis indicated that aggrecan was the predominant PG species. Northern blot analysis showed that the expression of link protein, type II collagen, and aggrecan mRNA transcripts was not modulated by CDMP treatment.**

Conclusion. **This study shows the presence of CDMP-1 and CDMP-2 in adult bovine and human articular cartilage. In addition, our in vitro data indicate that CDMPs 1 and 2 stimulate the metabolic activity of articular chondrocytes. Therefore, these signaling molecules may be contributing to the maintenance of the integrity of the joint surface.**

Cartilage-derived morphogenetic proteins 1 and 2 (CDMPs 1 and 2) are 2 recently discovered members of the transforming growth factor β (TGFβ) superfamily, which are essential for the formation of cartilaginous tissues during early limb development (1–3). The physiologic role of CDMP-1 has been established by identification of a null mutation in the *cdmp*-1 gene, which is characterized by skeletal abnormalities of the limbs and their synovial joints (Hunter-Thompson chondrodysplasia) (4). In addition, a specific expression pattern of CDMP-1 has been detected in areas of future joint spaces during embryonic development, implicating a role in the formation of the articular joint cavity (1,3). The primary function of CDMP-2 is unknown (1,2). Besides their involvement in joint formation, preliminary findings have shown the expression of CDMPs 1 and 2 in early postnatal cartilage, which suggests a possible role of CDMPs in the growth and maintenance of cartilaginous tissues (1,2). However, until this report, the presence of either of the CDMPs in adult and diseased cartilage had not been described.

Osteoarthritis (OA) is a degenerative joint disease involving both the articular cartilage and the un-

Dr. Erlacher's work was supported by an Erwin-Schrödinger Fellowship of the Austrian Society "Fonds zur Förderung der Wissenschaften."

Ludwig Erlacher, MD, Chee-Keng Ng, PhD: National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland; Frank P. Luyten, MD, PhD: National Institute of Dental Research, National Institutes of Health (current address: UZ Leuven, Pellenberg, Belgium); Robert Ullrich, MD, Sigurd Krieger, MS: University of Vienna, Vienna, Austria.

Address reprint requests to Ludwig Erlacher, MD, Clinic of Internal Medicine III, Department of Rheumatology, Währingergürtel 18-20, 1090 Vienna, Austria.

Submitted for publication April 30, 1997; accepted in revised form September 19, 1997.

derlying bone (5). A hallmark of OA is a progressive loss of extracellular cartilaginous matrix, leading to destruction of the joint surface (5). Trypsin treatment of cartilage explant cultures has been described previously as a useful approach to studying cartilage metabolism after tissue damage (6,7). Although it may not be the ideal model for OA, trypsin treatment depletes the cartilage matrix of its proteoglycans (PGs) (aggrecan and small PGs) and other noncollagenous matrix proteins (e.g., link protein), a condition which may be associated with an ongoing OA process (5,6). Trypsin-depletion of cartilage matrix leads to an initial decrease in PG biosynthesis, which is followed by a rapid recovery (6). This biosynthetic response may be interpreted as an attempt by the chondrocytes to repair the tissue damage.

Our data demonstrate the presence of CDMP-1 and CDMP-2 in adult bovine and human articular cartilage. Furthermore, using matrix-depleted bovine cartilage explant cultures as an in vitro model for cartilage injury, we show that CDMPs markedly enhance PG biosynthesis, thereby contributing to the restoration of the structural integrity of the tissue.

MATERIALS AND METHODS

Growth and differentiation factors. Recombinant CDMP-1 and CDMP-2 were expressed in *Escherichia coli*, purified, and refolded as described previously (8). Growth factor concentrations were estimated from ultraviolet absorbance at 280 nm. Protein was aliquoted and stored at −80°C.

Western immunoblotting. Cartilage explants were extracted in 4*M* guanidine HCl, 50 m*M* sodium acetate buffered at pH 7.2, in the presence of protease inhibitors, for 24 hours at 4°C, with shaking. Supernatants were precipitated with 30% trichloroacetic acid, and the pellets were washed twice with acetone. The precipitates were redissolved in 8*M* urea, 10 m*M* Tris, pH 7.8, and mixed with an equal volume of 2× Laemmli sample buffer. The samples were reduced with 2% β-mercaptoethanol and were separated on 4–20% Tris–glycine polyacrylamide gels (Novex, San Diego, CA).

Recombinant CDMP-1, CDMP-2, osteogenic protein 1 (OP-1), and bone morphogenetic protein 2 (BMP-2) (2.5 ng of protein/lane; Creative BioMolecules, Hopkinton, MA) were separated as above, under nonreducing conditions. Samples were blotted onto Immobilon-P membrane (Millipore, Bedford, MA) by using a GENIE electrophoretic blotter (Idea Scientific, Minneapolis, MN). Membranes were blocked for 30 minutes in blocking buffer, consisting of 10 m*M* Tris, pH 7.5, 0.9% NaCl, 0.05% Tween 20, and 4% bovine serum albumin (BSA), and probed with a polyclonal antibody to CDMP-1 and CDMP-2. Chicken polyclonal affinity-purified peptide antisera, prepared against the N-terminal sequences QGKRPSKNLKARC and HGKRHGKKSRLRC, which are specific for CDMP-1 and CDMP-2, respectively, were generous gifts from Pfizer Central Research (Groton, CT). Membranes were incubated for 60 minutes at room temperature with primary antisera at a 1:500 dilution in TBST (10 m*M* Tris, pH 7.9, 150 m*M* NaCl, 0.1% Tween 20) and 4% BSA, followed by washing in TBST and incubation with appropriate horseradish peroxidase–conjugated secondary antibody at a dilution of 1:50,000 for another 60 minutes (Jackson ImmunoResearch, Avondale, PA). Blots were developed by using the SuperSignal CL Substrate system (Pierce, Rockford, IL) for chemiluminescent detection, and then exposed to Kodak XAR-5 film.

Source of human articular cartilage, and immunohistochemistry analysis. Healthy articular cartilage was obtained postmortem from the tibial plateau of 5 young organ donors ages 24–42 years. The removal of cartilage from healthy donors was approved by the ethics committee of the University of Vienna. OA articular cartilage from 10 individuals ages 54–84 years was obtained at arthroplasty from the femoral heads of subjects who had sustained fractures of the femoral neck (Mankin grades 2 and 3).

Specimens of cartilage and subchondral bone were fixed immediately in 7.5% buffered formalin for 48 hours, decalcified with EDTA solution, followed by routine paraffin embedding, and processed for immunohistochemistry. Bovine articular cartilage samples were fixed in 4% paraformaldehyde for 48 hours, followed by paraffin embedding. The presence and distribution of CDMPs were determined by light microscopy, using the Supersensitive Kit (Biogenics, San Ramon, CA) according to the manufacturer's instructions. As a control, preimmune chicken serum was substituted for the CDMP 1 and 2 antibodies.

Cartilage explant cultures. Articular cartilage was obtained from the metacarpophalangeal joints of adult steers (ages 18–20 months), as previously described (9–11). The cartilage tissue was minced, washed 3 times in a chemically defined serum-free basal medium (BM), and redistributed in lots of 100–150 mg wet weight in 24-well culture plates (Costar, Cambridge, MA). To investigate the biosynthetic response in samples depleted of extracellular matrix, explants were digested with 5 μg/ml of trypsin (Sigma, St. Louis, MO) for 16 hours at 37°C. After incubation, medium was replaced with fresh BM, and the tissue was washed twice with BM containing 10% fetal bovine serum (FBS) to block the remaining trypsin activity (day 0). Cartilage explants were then maintained in BM supplemented with either CDMP-1, CDMP-2, 10% FBS, or 10% FBS plus CDMP-1 or CDMP-2 at 37°C in 95% air and 5% CO_2.

Growth factor concentration-dependent curves were obtained from cultures maintained in 10, 30, 100, and 300 ng/ml of CDMP-1 or CDMP-2 for 5 days. Cartilage samples which were not treated with trypsin served as controls. Culture medium was changed daily. The medium-to-tissue ratio (1.5 ml/100 mg wet weight) was kept constant throughout the culture period (9–11). All experiments were performed using a chemically defined BM as described (8).

Measurement of PG biosynthesis. Rates of ^{35}S-sulfate incorporation into PGs were determined on days 0, 1, and 7 after trypsin treatment, as described previously (10). Briefly, tissue samples were labeled in identical aliquots of 1.0 ml of BM with 20 μCi/ml of ^{35}S-sulfate for 4 hours at 37°C. After radiolabeling, each culture was washed twice with ice-cold buffer (10 m*M* EDTA, 0.1*M* sodium phosphate buffered at pH 6.5) and digested with proteinase K (1 mg/ml; 80°C for 16 hours) in 1 ml of the sodium phosphate wash buffer. Newly synthesized ^{35}S-labeled macromolecules of tissue digests were determined after removal of unincorporated isotope by use of

Sephadex G-25 (PD-10; Pharmacia Biotech, Piscataway, NJ) gel chromatography in 4*M* guanidine HCl, 50 m*M* sodium acetate buffered at pH 7.2, containing 0.5% Triton X-100. The radioactivity for each sample was normalized to the respective hydroxyproline mass using a previously described method (11).

PG analysis. Articular cartilage explants were grown in BM, with or without CDMP-1, CDMP-2, and/or FBS, as described above. At the end of the culture period, samples were radiolabeled with ^{35}S-sulfate (20 μCi/ml) for 4 hours. The radiolabeling medium was removed and stored at 4°C. Cartilage explants were extracted in 4*M* guanidine HCl, 50 m*M* sodium acetate buffered at pH 7.2, in the presence of protease inhibitors at 4°C for 48 hours, with shaking (10). After the removal of guanidine HCl, tissue was then extracted with 0.5*M* NaOH at 4°C for 48 hours.

Medium and tissue extracts were separately analyzed for ^{35}S-labeled macromolecules using gel chromatography on PD-10 columns. The void volume for all samples was collected and an aliquot was further analyzed using a Sephacryl S-500 HR column (1 × 30 cm; Pharmacia Biotech), preequilibrated in 4*M* guanidine HCl, 0.5% Triton X-100 in 50 m*M* sodium acetate buffer, pH 7.2. Fractions of 0.4 ml/minute were collected and analyzed for radioactivity. The hydrodynamic size of newly synthesized glycosaminoglycan (GAG) chains derived from alkaline degradation of PGs was determined using a Superose 6 column (1 × 30 cm; Pharmacia Biotech) equilibrated with 4*M* guanidine HCl, 50 m*M* sodium acetate buffer, pH 7.2, 0.5% Triton X-100 at a flow rate of 0.4 ml/minute (11). Radioactivity in each fraction was determined by liquid scintillation counting. The recovery of radioactivity from each column was ~95%.

Determination of DNA and GAG content. DNA content from proteinase K–digested explant cultures was determined for days 0, 1, and 7 cultures by use of bisbenzimide (Hoechst 33258; Sigma) (12). GAG content was measured using a densitometric assay by precipitation with Safranin O as described previously (11).

RNA isolation and Northern blot analysis. Total RNA was extracted using a modified acidic guanidine–phenol–chloroform method (13). For Northern blot analysis, equal amounts of total RNA (5 μg) were electrophoresed on 1.2% agarose–formaldehyde gels and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH). The blots were prehybridized for 30 minutes at 68°C in hybridization buffer (Express Hyb; Clontech, Palo Alto, CA) and hybridization was performed for 1 hour at 68°C in the same buffer with ^{32}P-labeled probes. Probes included a mouse complementary DNA (cDNA) encoding for aggrecan, a rat cDNA for type II collagen, and a human cDNA for link protein. A cDNA probe for GAPDH was used as a control.

After hybridization, the filters were washed 4 times in 2× saline–sodium citrate (SSC), 0.05% sodium dodecyl sulfate (SDS), and twice in 0.2× SSC, 0.1% SDS at room temperature for 10 minutes. The blots were first exposed to Kodak XAR-5 film at −70°C for up to 12 hours, and then were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The amounts of transcripts detected were normalized to GAPDH levels.

Statistical analysis. Statistical analysis was performed using the Mann-Whitney U test. Statistical significance was defined as a *P* value < 0.05.

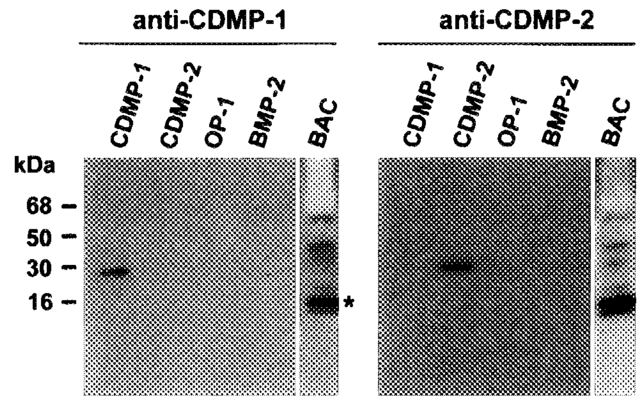

Figure 1. Characterization of polyclonal antisera and detection of cartilage-derived morphogenetic proteins 1 and 2 (CDMPs 1 and 2) in bovine articular cartilage (BAC) extracts. Polyclonal antibodies were raised against synthetic peptides corresponding to the N-terminal domain of the mature region of CDMP-1 and CDMP-2, respectively, as described in Materials and Methods. The specificity of these antibodies was tested by Western blot analysis using chemiluminescence. These antisera detected CDMP-1 and CDMP-2 (*) in 4*M* guanidine HCl extracts of bovine articular cartilage under reduced conditions. OP-1 = osteogenic protein 1; BMP-2 = bone morphogenetic protein 2. Molecular weight markers are shown at the left.

RESULTS

Presence of CDMP-1 and CDMP-2 in adult bovine and human articular cartilage. Western blot analysis demonstrated that the polyclonal antibodies against CDMPs 1 and 2 exclusively recognized their respective recombinant protein and not other structurally related proteins (OP-1 and BMP-2) (Figure 1). In addition, CDMP-1 and CDMP-2 were detected in extracts of bovine articular cartilage (Figure 1).

Immunohistochemical staining of cultured bovine articular cartilage specimens revealed the presence of the CDMPs in most of the chondrocytes throughout the tissue (Figure 2). Interestingly, trypsin-treated samples showed an increased staining of the extracellular matrix (Figures 2B and C), which was not observed in the controls (Figure 2A) or the non–trypsin-treated samples (Figures 2D–F).

Immunostaining of healthy articular cartilage samples obtained from 5 different adult organ donors showed discrete staining for CDMP-1 and CDMP-2, predominantly in the upper one-third layer of the cartilage sections (Figures 3B and C). Only a few cells in the deeper layers stained for CDMPs. The staining patterns for CDMP-1 and CDMP-2 appeared to overlap. Pronounced staining for CDMPs, associated with cell clusters, and weak staining in the matrix, was observed in OA samples (10 patients studied; see Materials and Methods), preferentially localized in the histologically

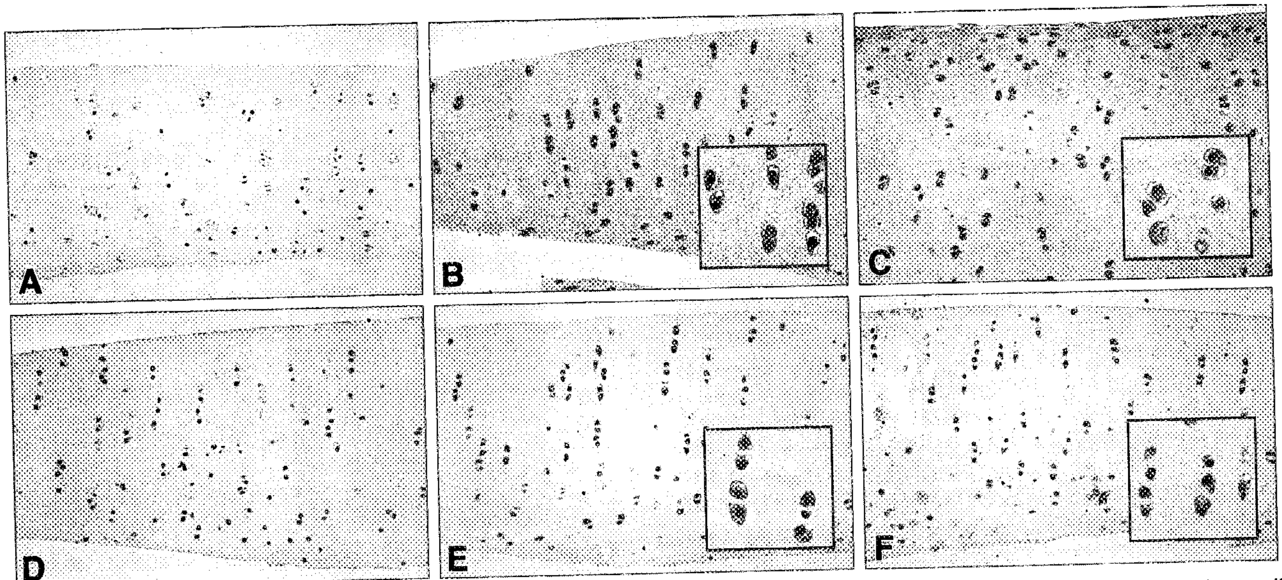

Figure 2. Immunohistochemical staining for cartilage-derived morphogenetic proteins 1 and 2 (CDMPs 1 and 2) in bovine articular cartilage. Bovine articular cartilage explants were stained for CDMP-1 (**B** and **E**) and CDMP-2 (**C** and **F**) using specific polyclonal antisera. Trypsin-treated (**A–C**) and non–trypsin-treated (**D–F**) samples were collected after 24 hours of in vitro culture, fixed, and stained as described in Materials and Methods. Control samples (**A** and **D**) with nonimmune sera do not show any significant background staining. (Original magnification × 100; insets × 400.)

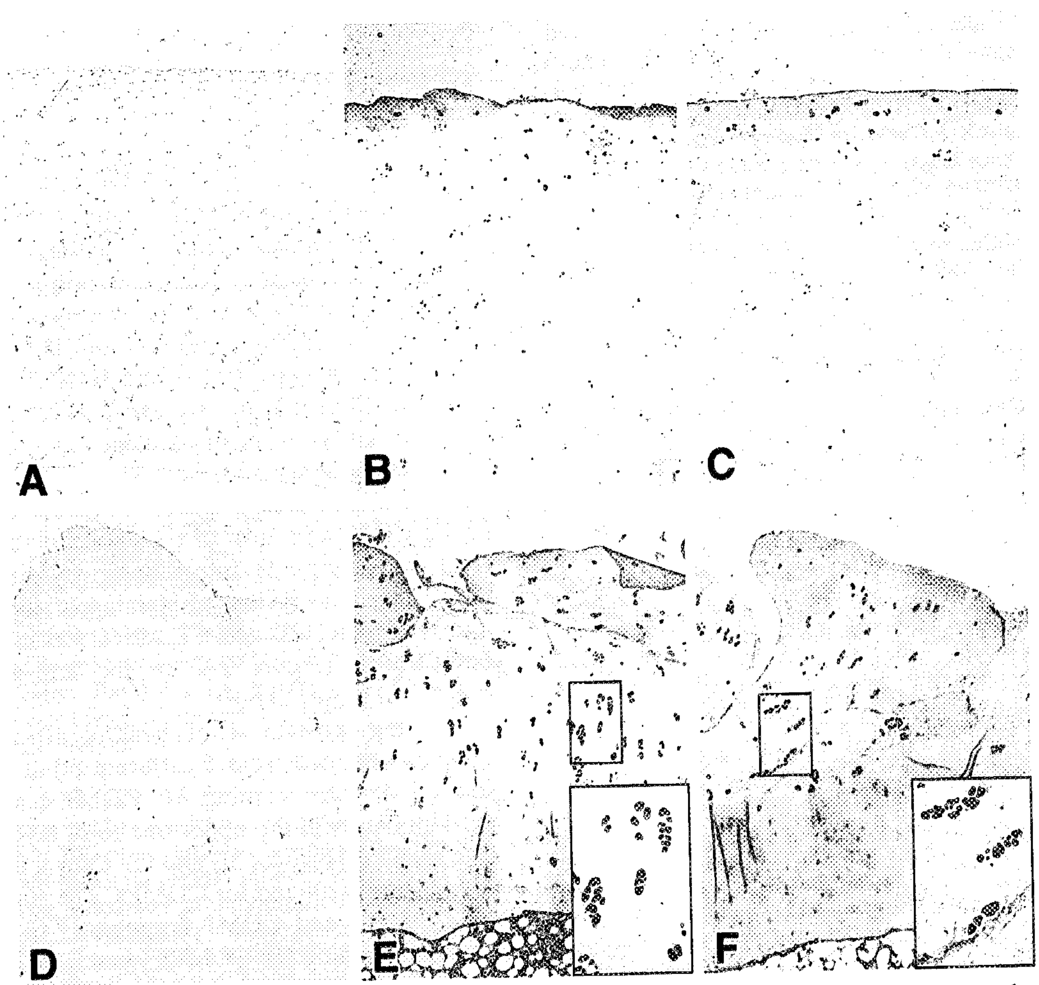

Figure 3. Immunohistochemical staining for cartilage-derived morphogenetic proteins 1 and 2 (CDMPs 1 and 2) in healthy and osteoarthritic (OA) adult human articular cartilage. Specimens were stained for CDMPs 1 and 2 using specific polyclonal antibodies. **A** and **D,** Control samples incubated with nonimmune sera, showing no immunohistochemical staining. **B** and **C,** Healthy adult cartilage, showing the presence of CDMPs 1 and 2. **E** and **F,** OA cartilage, showing pronounced staining for CDMPs 1 and 2. (Original magnification × 100; insets × 400.)

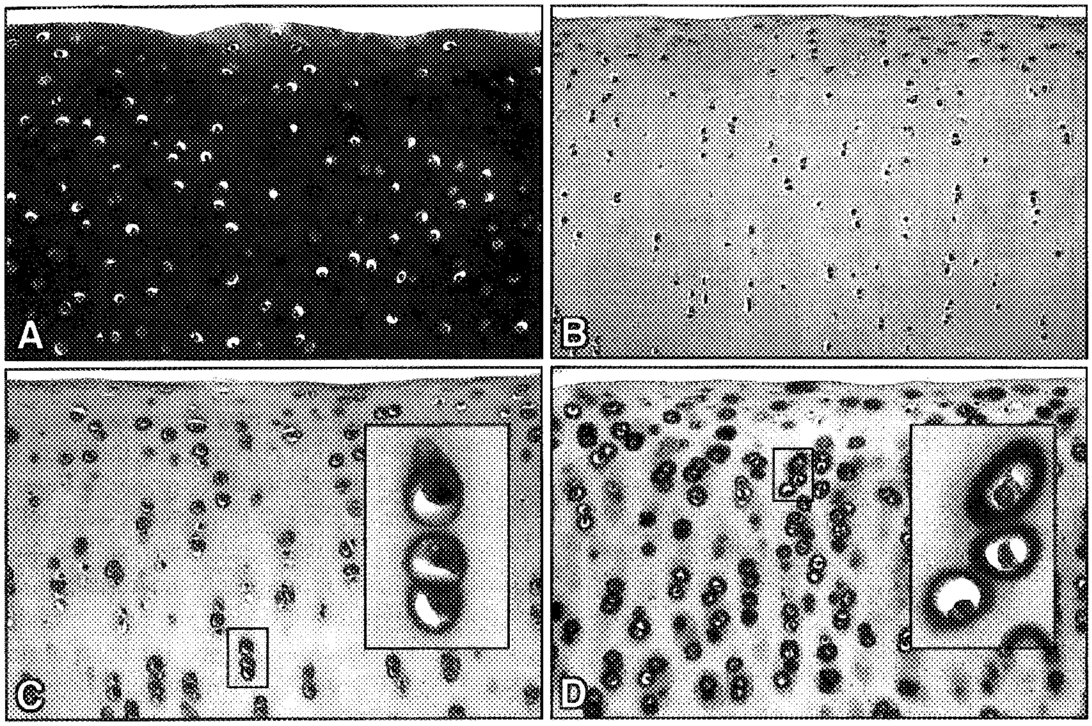

Figure 4. Toluidine blue staining of trypsin-treated bovine articular cartilage explants. **A,** Nontrypsinized adult bovine articular cartilage, showing uniform staining of the matrix. **B,** Cartilaginous tissue treated for 16 hours with 5 μg/ml of trypsin, showing depletion of the extracellular matrix as displayed by the lack of metachromatic staining (day 0). **C,** Cartilage-derived morphogenetic protein 1 (CDMP-1; 100 ng/ml)–treated tissue, showing a halo of newly synthesized proteoglycans surrounding the chondrocytes after 1 day. **D,** CDMP-1 (100 ng/ml)–treated tissue after 7 days in culture, showing an area of increased toluidine blue staining of newly synthesized matrix surrounding the cells. (Original magnification × 100; insets × 400.)

most-affected zones of the articular cartilage (Figures 3E and F). Specimens incubated with nonimmune sera did not reveal any staining (Figures 3A and D).

Effects of trypsin treatment on matrix depletion. Adult bovine cartilage tissue was treated with trypsin (5 μg/ml), and histologic evaluation was performed using toluidine blue, which stains for cationic macromolecules. Nontrypsinized control tissue showed a uniform staining of the matrix, indicating the presence of PGs and other negatively charged macromolecules (Figure 4A). Immediately after treatment with trypsin, little or no staining was observed, indicating a nearly complete removal of matrix PGs (Figure 4B). Measurement of the GAG content, as determined by Safranin O assay, confirmed the histologic findings with low or undetectable levels of GAGs in trypsinized samples (<250 ng). This was in contrast to nontrypsinized control samples, which contained a mean ± SEM of 143 ± 19 μg of GAG/mg of hydroxyproline.

After 1 day in culture in BM, with or without the addition of CDMPs or FBS, toluidine blue–stained macromolecules were observed surrounding chondrocytes (Figure 4C). These areas of staining increased in size over the 7-day culture period, which suggests matrix replenishment (Figure 4D).

Increased PG synthesis in trypsin-treated explant cultures induced by CDMPs 1 and 2. CDMP-1 and CDMP-2 increased ^{35}S-sulfate incorporation into PGs equally, in a concentration-dependent manner (Figure 5). The optimal concentration of CDMPs 1 and 2 was 100 ng/ml. This concentration was used for all subsequent investigations.

On day 0, trypsin treatment resulted in a reduced level of PG biosynthesis, as determined by ^{35}S-sulfate incorporation into macromolecules, compared with the nontrypsinized samples ($P < 0.03$) (Figure 6).

After 1 day in culture, this down-regulation in the biosynthetic activity was followed by an immediate recovery of PG synthesis, whereupon the rate of PG synthesis was similar to that of the nontrypsinized control cultures. The addition of CDMP-1, CDMP-2, and/or FBS did not increase the rate of ^{35}S-labeled PG synthesis compared with cultures maintained in BM alone at day 1 (Figure 6).

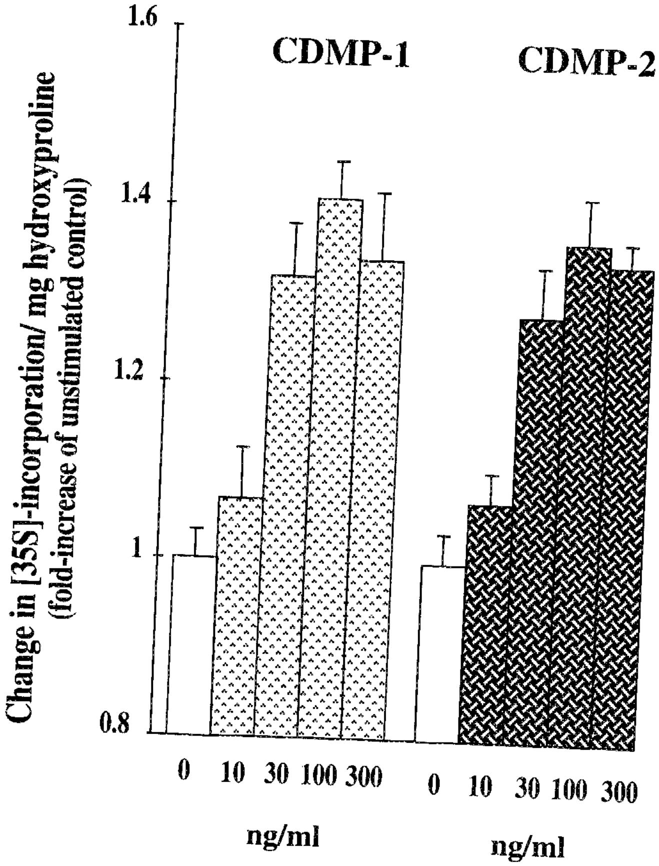

Figure 5. Concentration-dependent effect of cartilage-derived morphogenetic proteins 1 and 2 (CDMPs 1 and 2) on proteoglycan (PG) synthesis in cartilage explants. Quadruplicate cultures of bovine cartilage tissue were maintained for 5 days with 10, 30, 100, and 300 ng/ml of CDMP-1 or CDMP-2 in basal medium (BM). Tissue cultured in BM alone served as unstimulated controls. Cartilage explant cultures were labeled with ^{35}S-sulfate for 4 hours, and the incorporated radiolabel was calculated per milligram of hydroxyproline. Bars show the times increase in ^{35}S-sulfate incorporation into PGs over unstimulated controls. Values are the mean and SEM of 1 experiment (from 2 similar experiments).

Cartilage explants cultured for 7 days in the presence of CDMP-1 or CDMP-2 showed a significant increase in the rate of ^{35}S-labeled PG biosynthesis compared with BM alone ($P < 0.03$) (Figure 6). This increase in PG synthesis was similar to the rates obtained from tissue maintained in FBS-supplemented medium (Figure 6). Interestingly, the addition of CDMP-1 or CDMP-2 to cultures maintained in FBS resulted in a further 38–48% increase in ^{35}S incorporation.

The addition of optimum concentrations of CDMP-1 (100 ng/ml) to cartilage explants cultured in the presence of optimum concentrations of CDMP-2 did not show any additional or synergistic effect on ^{35}S-labeled PG synthesis (results not shown). The latter findings suggest that both CDMPs use the same signaling pathway in regulating PG synthesis in the explant cultures.

Analysis of newly synthesized PGs in cartilage explant cultures maintained in the presence of CDMPs 1 and 2. Newly synthesized macromolecules from cartilage tissue extracted with guanidine HCl were analyzed on a Sephacryl S-500 HR column as described in Materials and Methods. The hydrodynamic profile from day-0 trypsin-treated cultures indicated 2 distinct ^{35}S-labeled PG populations, one corresponding to large PGs, which eluted with a peak K_d of 0.09, and the other representing small PGs, with a peak K_d of 0.55 (Figure 7A). The proportions of these 2 PG species were 80% and 20%, respectively (Table 1). In contrast, the hydrodynamic profile of the ^{35}S-labeled macromolecules extracted from nontrypsinized tissue mainly consisted of the large PG species, which accounted for 95% of newly synthesized PGs (Figure 7A). The small PG species eluted off the column as the lagging shoulder of the profile, and represents 5% of the total ^{35}S incorporated into macromolecules (Table 1). The recovery from trypsin treatment in cartilage explants after 1 day in culture was characterized by an increased proportion of the large PG species over that of the small species (Figure 7A), which eluted with a peak K_d of 0.51 (Table 1).

Analysis of the hydrodynamic size of the radiolabeled macromolecules 7 days after trypsin treatment demonstrated the presence of mainly the large PG species, similar to that observed for nontrypsinized tissue (Figure 7B). The addition of CDMP-1 or CDMP-2 did not alter the size of the newly synthesized PGs (Figure 7B) or the proportion of ^{35}S radioactivity in the 2 PG species over the 7-day culture period (Table 1).

Analysis of culture media showed that trypsin treatment of cartilage tissue resulted in an increased release of ^{35}S-labeled PGs into culture medium (14%) compared with controls (3%). Over the study period, the percentage of newly synthesized ^{35}S-labeled PGs found in culture medium gradually decreased to the amount observed for the nontrypsinized sample (results not shown).

To determine if the recovery from matrix depletion had any effect on the size of the GAG chains, we analyzed the newly synthesized GAGs derived from alkaline reduction by molecular sieve chromatography using a Superose 6 column. The ^{35}S-labeled GAGs in trypsin-treated and control cultures were found to have

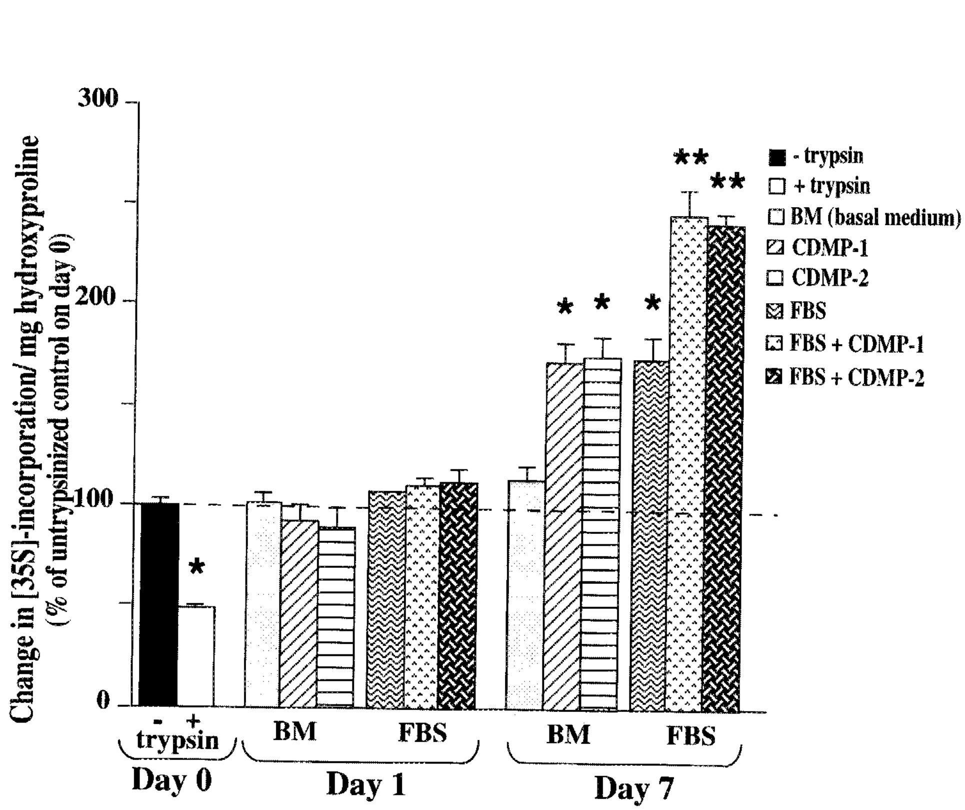

Figure 6. ^{35}S-sulfate incorporation into proteoglycans (PGs) in trypsin-treated bovine articular explant cultures. Quadruplicate cultures of cartilage explants were depleted of extracellular matrix by digestion with 5 μg/ml of trypsin for 16 hours. The next day (day 0), the medium was replaced with basal medium (BM) and the explants were maintained in a chemically defined BM supplemented with either cartilage-derived morphogenetic protein 1 (CDMP-1; 100 ng/ml), CDMP-2 (100 ng/ml), 10% fetal bovine serum (FBS), or 10% FBS plus CDMP-1 or CDMP-2 for up to 7 days. Nontrypsinized samples (day 0) served as controls. Cartilage explant cultures were labeled with ^{35}S-sulfate for 4 hours, and the incorporated radiolabel was calculated per milligram of hydroxyproline. Values are given as the percentage of ^{35}S-sulfate incorporation into PGs compared with nontrypsinized cartilage tissue. Bars show the mean and SEM. Experiments were repeated twice. Day 0 * = $P < 0.03$ versus nontrypsinized controls; day 7 * = $P < 0.03$ versus BM and ** = $P < 0.03$ versus BM, CDMP-1, CDMP-2, and FBS.

a peak K_d of 0.53 (Figure 8). The presence of CDMP-1 and CDMP-2 did not result in a detectable change in the hydrodynamic size of the PG side chains (Figure 8).

Lack of effect of CDMPs on DNA and protein content. The DNA content per milligram of hydroxyproline was unchanged throughout the 7-day culture period for samples maintained in BM, with or without CDMP-1, CDMP-2, and FBS (mean ± SEM 49 ± 5 μg of DNA/mg of hydroxyproline). The overall protein synthesis, as measured by ^{3}H-leucine incorporation into total protein synthesis, was unaffected by the addition of CDMPs to trypsin-treated cartilage explants (10,500 ± 1,500 counts per minute/mg of hydroxyproline).

Lack of effect of CDMPs on gene expression of chondrogenic markers. Matrix-depleted bovine articular explants were cultured for up to 5 days in the presence of BM, CDMP-1, CDMP-2, or FBS. Nontrypsinized cartilage tissue served as a control. Northern blot analysis for type II collagen (5.3 kb), aggrecan (8.5 kb), and link protein (3 kb and 5 kb) did not reveal any appreciable increase in the mRNA levels for each of the chondrogenic markers over the study period (Figure 9).

DISCUSSION

We report that both CDMP-1 and CDMP-2 stimulate the biosynthetic activity of chondrocytes in an in vitro model of matrix replacement, using trypsin-treated bovine articular cartilage explants. The presence of the CDMPs in adult bovine and human articular cartilage suggests a possible role of the CDMPs in the maintenance and regeneration of the articular surface.

CDMP-1 and CDMP-2 are 2 highly related growth factors and are part of a subgroup of morpho-

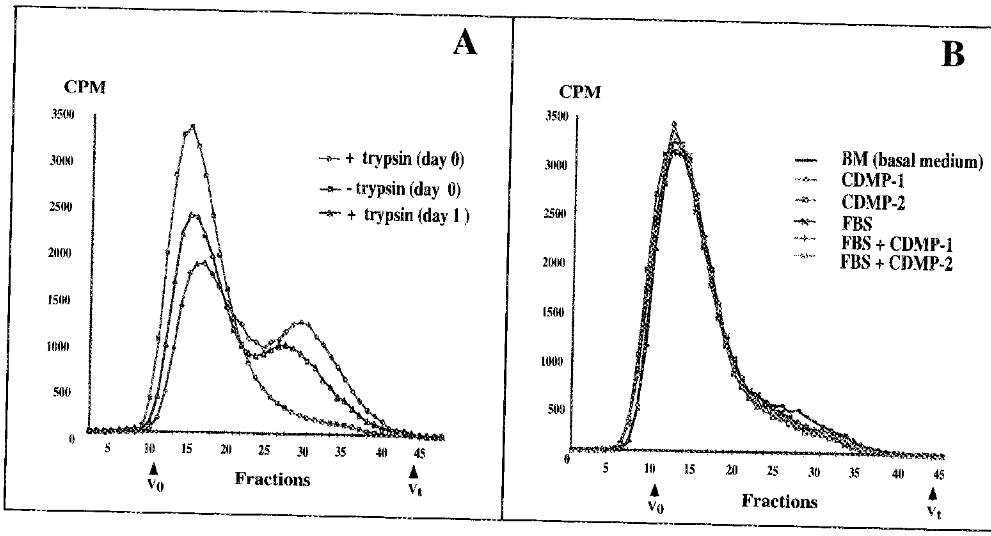

Figure 7. Effect of cartilage-derived morphogenetic proteins 1 and 2 (CDMPs 1 and 2) and fetal bovine serum (FBS) on the hydrodynamic size of newly synthesized macromolecules of trypsin-treated cartilage explant cultures. Quadruplicate cultures of cartilaginous tissue were digested with 5 μg/ml of trypsin for 16 hours. The next day, medium was replaced with basal medium (BM) (day 0), and the explants were maintained in a chemically defined BM supplemented with either CDMP-1 (100 ng/ml), CDMP-2 (100 ng/ml), 10% FBS, or 10% FBS plus CDMP 1 or 2 for **A**, 0 or 1 day or **B**, 7 days. Nontrypsinized samples served as controls. Analysis of the hydrodynamic size of ^{35}S-labeled newly synthesized macromolecules was performed on a Sephacryl S-500 HR column equilibrated in 4*M* guanidine HCl, 0.05*M* sodium acetate, pH 7.2, with 0.5% Triton X-100. Plots were obtained after adjustment of the samples to equal counts. V_0 = void volume; V_t = total volume.

Table 1. Synthesis of large and small PG species in trypsin-treated adult bovine articular cartilage*

Culture condition	Large PGs (%)	Small PGs (%)
Day 0		
Trypsin		
Present	80	20
Absent	95	5
Day 1		
Basal medium	81	19
CDMP-1	83	17
CDMP-2	82	18
FBS	81	19
+ CDMP-1	81	19
+ CDMP-2	81	19
Day 7		
Basal medium	91	9
CDMP-1	94	6
CDMP-2	93	7
FBS	92	8
+ CDMP-1	93	7
+ CDMP-2	93	7

* Trypsin-treated cartilage explant cultures were radiolabeled for 4 hours with ^{35}S-sulfate and then extracted with 4*M* guanidine HCl for 48 hours. Extracts were analyzed by Sephacryl S-500 HR column gel chromatography as described in Materials and Methods. The relative proportion of large proteoglycans (PGs) to small PGs was calculated as a percentage of total radioactivity. CDMP = cartilage-derived morphogenetic protein; FBS = fetal bovine serum.

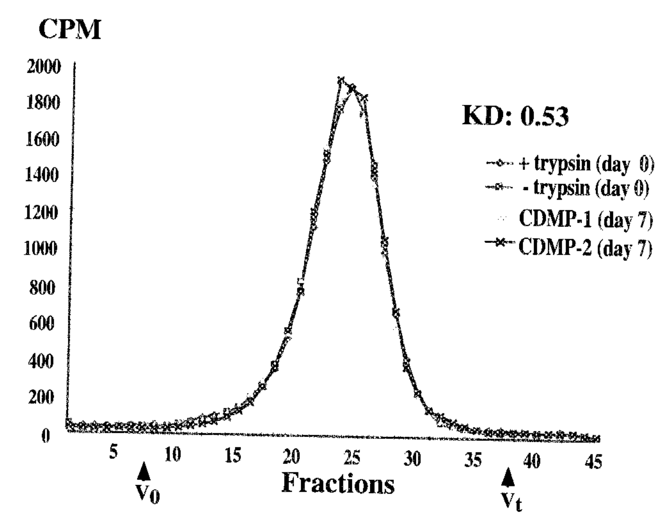

Figure 8. Molecular sieve chromatography of the newly synthesized glycosaminoglycan (GAG) chains in cartilage explant cultures. ^{35}S-sulfate–labeled material was extracted from trypsin-treated and non–trypsin-treated cartilage explant cultures and then treated with 0.5*M* NaOH. GAG chains were analyzed by molecular sieve chromatography on Superose 6 preequilibrated in 4*M* guanidine HCl, 0.05*M* sodium acetate, pH 7.2, with 0.5% Triton X-100. CDMP = cartilage-derived morphogenetic protein; KD = distribution coefficient; V_0 = void volume; V_t = total volume.

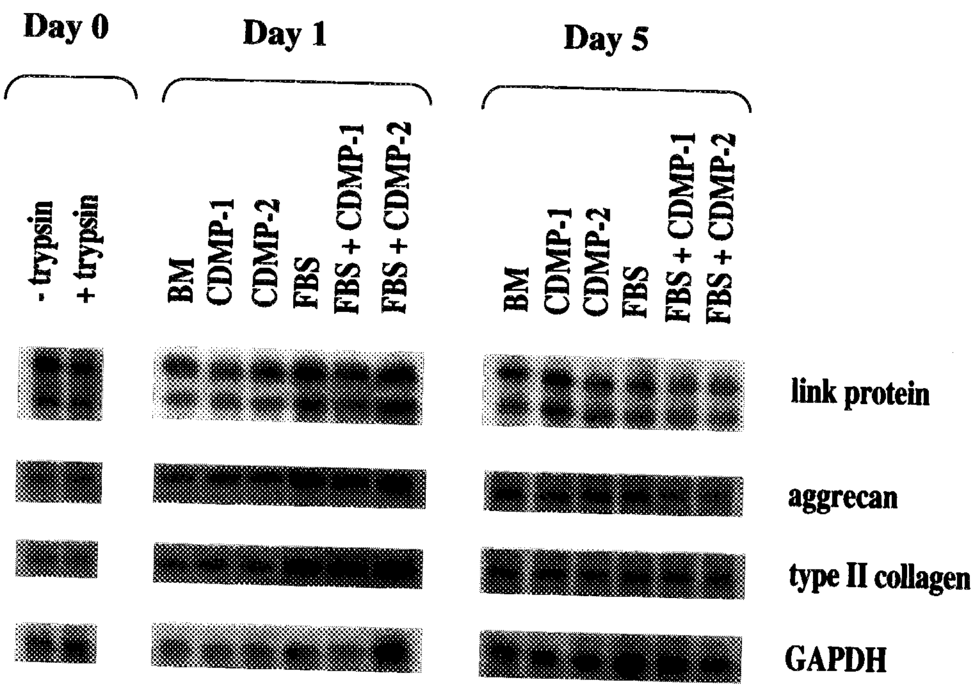

Figure 9. Northern blot analysis of link protein, aggrecan, and type II collagen expression in trypsin-treated cartilage explant cultures. Trypsin-treated cartilage tissue was cultured in basal medium (BM), with or without cartilage-derived morphogenetic protein 1 (CDMP-1; 100 ng/ml), CDMP-2 (100 ng/ml), 10% fetal bovine serum (FBS), or 10% FBS plus CDMP-1 or CDMP-2 for up to 5 days. Nontrypsinized tissue served as controls. Total RNA from each culture (5 μg) was separated on 1.2% agarose–formaldehyde gels, transferred, and subsequently hybridized with complementary DNA probes for link protein, aggrecan, and type II collagen as described in Materials and Methods. GAPDH expression levels are shown to verify equal loading of messenger RNA (bottom lane).

gens within the BMP family (1–3). Members of this family, BMPs 2, 4, 6, and 7 (also called osteogenic protein 1), along with CDMPs 1 and 2, are expressed in and around the appendicular skeletal elements and play important functions in the cellular differentiation during limb development (1–3,14–17). Despite their close homology at the protein level, CDMPs 1 and 2 are 2 distinct growth factors with apparently different biologic roles (1,2). We have reported the expression of CDMP-1 and CDMP-2 mRNA in newborn calf articular chondrocytes, suggesting the presence of these 2 growth factors in postnatal cartilaginous tissues (1). Western analysis and immunohistochemical staining, using polyclonal antibodies specific for each CDMP, revealed that CDMPs 1 and 2 have similar distributions in adult bovine and human articular cartilage. This localization pattern provides support for the notion that CDMPs 1 and 2 are not only important for skeletal development, but they may also be involved in the maintenance of normal cartilage and regenerative responses in diseased tissue. Currently, the presence of other members of the BMP family in adult articular cartilage is not known and is the subject of an ongoing investigation.

It has been shown, by use of a variety of in vitro models, that members of the BMP family promote chondrogenesis (18–20), enhance cartilage matrix synthesis (10,21,22), and support the reexpression of the cartilage phenotype (23,24). Therefore, the role of the CDMPs in this microenvironment might be to contribute to any of the above-mentioned functions. We have addressed the potential contribution of CDMPs to the maintenance of the integrity of the articular surface, using the explant culture model after matrix depletion (6,7). In this in vitro model, PGs were removed by trypsin digestion. Trypsin digestion of cartilage leaves the collagen network mostly intact, and the production of new PG matrix is therefore a process of tissue replenishment. It is noteworthy that in this regard, most forms of OA diseases involve damage to the collagen network. Analysis of PGs following treatment with trypsin demonstrated a change in the ^{35}S-labeled PG hydrodynamic profile. In normal cartilage cultures, the large

PG (aggrecan) represents ~95% of the total newly synthesized ^{35}S-labeled PGs, whereas cartilage explants damaged by trypsin showed an increased proportion of the small PG species (~20%). Similar metabolic findings with respect to PG synthesis in trypsin-treated cartilage explants have been reported previously (6). However, unlike the previous study, we did not detect any appreciable change in the size of the GAG side chains. This may be due to greater tissue damage since different concentrations of trypsin were used (5 μg/ml versus 20 μg/ml).

Addition of recombinant CDMPs 1 and 2 to the media of matrix-depleted explant cultures resulted in a stimulation of PG biosynthesis. This increase in ^{35}S-sulfate incorporation into newly synthesized PGs was mainly due to an up-regulation of aggrecan and did not result from an increase in the size of the GAG side chains. Interestingly, the higher level of aggrecan synthesis was not correlated with an increase in the steady-state mRNA level for this PG, as evaluated by Northern blot analysis. It was previously reported that the steady-state mRNA levels for aggrecan and type II collagen in chondrocytes cultured in the presence of BMP-2 were unaffected over a 1-week study period (25). One possible explanation for these observations is that BMPs/CDMPs may influence translational mechanisms, glycosylation, or secretion of matrix molecules. Alternatively, mRNA up-regulation of these chondrogenic markers may not be possible under the established culture conditions, or it may not be detectable and might reflect limitations of the detection method.

The stimulatory effects of CDMP-1 or CDMP-2 on PG biosynthetic activity were synergistic with growth factors found in FBS. Interestingly, OP-1, a structurally related member of the BMP family, was reported to exert an additive stimulatory effect on PG synthesis in fetal and adolescent human chondrocytes grown in alginate bead cultures (22). This suggests that there may indeed be a specific BMP (or TGFβ superfamily) signaling pathway that regulates matrix synthesis.

Coculturing of cartilage explants in the presence of optimum concentrations of CDMP-1 and optimum concentrations of CDMP-2 did not result in any additional or synergistic effect on the ^{35}S-labeled PG synthesis. This indicates that both ligands may recognize a common receptor. Members of the TGFβ superfamily, which include BMPs and CDMPs, induce their biologic effects through binding to heteromeric complexes of type I and type II serine-threonine kinase receptors (26–28). At present, the receptor complex(es) for CDMPs 1 and 2 in mature chondrocytes from articular cartilage is not known. Binding studies performed by Nishitoh et al (29) and our group (8) recently revealed the binding and signaling of both CDMPs to a BMP receptor type IB–BMP receptor type II complex.

Finally, it is noteworthy, that CDMPs 1 and 2 appear to be coexpressed in both normal and OA articular cartilage. This overlapping expression pattern may indicate that the biologic role of CDMP-1 and CDMP-2 in this tissue is complementary. Alternatively, their function may be redundant, providing a compensatory mechanism for potential mutational damage.

In conclusion, this study showed the presence of CDMP-1 and CDMP-2 in adult bovine and human articular cartilage and characterized their stimulatory effects on chondrocyte metabolism, using an in vitro model of matrix damage. These data and the preferential stimulation of chondrogenic differentiation over osteogenesis (8) may provide a scientific basis from which to further explore the possible physiologic and therapeutic role of CDMPs in cartilage repair.

ACKNOWLEDGMENTS

Drs. Ng and Erlacher are grateful to Prof. M. Yanagishita for his outstanding mentorship and for his critical reading of the manuscript. We thank Drs. L. Fisher, T. Thomas, and S. Dieudonné for their helpful comments, Drs. John McCartney and Herman Oppermann (Creative BioMolecules, Hopkinton, MA) for providing the recombinant growth factors, and Ms D. Carbott for her excellent technical expertise.

REFERENCES

1. Chang SC, Hoang B, Thomas JT, Vukicevic S, Luyten FP, Ryba NJP, et al. Cartilage-derived morphogenetic proteins: new members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. J Biol Chem 1994;269:28227–34.
2. Luyten FP. Cartilage-derived morphogenetic proteins: key regulators in chondrocyte differentiation? Acta Orthop Scand 1995; Suppl 266:51–4.
3. Storm EE, Huynh TV, Copeland NG, Jenkins NA, Kingsley DM, Lee SJ. Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. Nature 1994;368: 639–43.
4. Thomas JT, Lin K, Nandedkar M, Camargo M, Cervenka J, Luyten FP. A human chondrodysplasia due to a mutation in a TGF-beta superfamily member. Nat Genet 1996;12:315–7.
5. Poole AR. Imbalances of anabolism and catabolism of cartilage matrix components in osteoarthritis. In: Kuettner KE, Goldberg VM, editors. Osteoarthritic disorders. Rosemont (IL): American Academy of Orthopaedic Surgeons; 1995. p. 247–60.
6. Bartholomew JS, Handley CJ, Lowther DA. The effects of trypsin treatment on proteoglycan biosynthesis by bovine articular cartilage. Biochem J 1985;227:429–37.

7. Verbruggen G, Luyten FP, Veys EM. Repair function in organ cultured human cartilage: replacement of enzymatically removed proteoglycans during long-term organ culture. J Rheumatol 1985; 12:665–74.
8. Erlacher L, McCartney J, Piek E, ten Dijke P, Yanagishita M, Oppermann H, et al. Cartilage-derived morphogenetic proteins and osteogenic protein-1 differentially regulate osteogenesis. J Bone Miner Res. In press 1998.
9. Handley CJ, Ng CK, Curtis CJ. Introduction: short- and long-term explant culture of cartilage. In: Maroudas A, Kuettner K, editors. Methods in cartilage research. London: Academic Press, 1990. p. 105–8.
10. Luyten FP, Yu YM, Yanagishita M, Vukicevic S, Hammonds RG, Reddi AH. Natural bovine osteogenin and recombinant human bone morphogenetic protein-2B are equipotent in the maintenance of proteoglycans in bovine articular cartilage explant cultures. J Biol Chem 1992;267:3691–5.
11. Schafer SJ, Luyten FP, Yanagishita M, Reddi AH. Proteoglycan metabolism is age related and modulated by isoforms of platelet-derived growth factor in bovine articular cartilage explant cultures. Arch Biochem Biophys 1993;302:431–8.
12. Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 1980;102:344–52.
13. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
14. Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, et al. Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. J Biol Chem 1992;267:20352–62.
15. Storm EE, Kingsley DM. Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family. Development 1996;122:3969–79.
16. Luo G, Hofmann C, Bronckers ALJJ, Sohocki M, Bradley A, Karsenty G. BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes Dev 1995;9:2808–20.
17. Lyons KM, Hogan BLM, Robertson EJ. Colocalization of BMP-7 and BMP-2 rnRNAs suggests that these factors cooperatively mediate tissue interactions during murine development. Mech Dev 1995;50:71–8.
18. Vukicevic S, Luyten FP, Reddi AH. Stimulation of the expression of osteogenic and chondrogenic phenotypes in vitro by osteogenin. Proc Natl Acad Sci U S A 1989;86:8793–7.
19. Carrington JL, Chen P, Yanagishita M, Reddi AH. Osteogenin (bone morphogenetic protein-3) stimulates cartilage formation by chick limb bud cells in vitro. Dev Biol 1991;146:406–15.
20. Chen P, Carrington JL, Hammonds RG, Reddi AH. Stimulation of chondrogenesis in limb bud mesoderm cells by recombinant human bone morphogenetic protein 2B (BMP-2B) and modulation by transforming growth factor beta 1 and beta 2. Exp Cell Res 1991;195:509–15.
21. Luyten FP, Chen P, Paralkar V, Reddi AH. Recombinant bone morphogenetic protein-4, transforming growth factor-beta 1, and activin A enhance the cartilage phenotype of articular chondrocytes in vitro. Exp Cell Res 1994;210:224–9.
22. Flechtenmacher J, Huch K, Thonar EJ-MA, Mollenhauer JA, Davies SR, Schmid TM, et al. Recombinant human osteogenic protein 1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum 1996;39:1896–904.
23. Harrison ET Jr, Luyten FP, Reddi AH. Osteogenin promotes reexpression of cartilage phenotype by dedifferentiated articular chondrocytes in serum-free medium. Exp Cell Res 1991;192: 340–5.
24. Harrison ET Jr, Luyten FP, Reddi AH. Transforming growth factor-beta: its effect on phenotype reexpression by dedifferentiated chondrocytes in the presence and absence of osteogenin. In Vitro Cell Dev Biol 1992;28A:445–8.
25. Sailor LZ, Hewick RM, Morris ER. Recombinant human bone morphogenetic protein-2 maintains the articular chondrocyte phenotype in long-term culture. J Orthop Res 1996;14:937–45.
26. Ten Dijke P, Miyazono K, Heldin CH. Signaling via hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors. Curr Opin Cell Biol 1996;8:139–45.
27. Kingsley DM. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev 1994;8:133–46.
28. Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, Smith JC, et al. Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. J Cell Biol 1995;130:217–26.
29. Nishitoh H, Ichijo H, Kimura M, Matsumoto T, Makishima F, Yamaguchi A, et al. Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5. J Biol Chem 1996;271:21345–52.